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Abstract

***In vitro* culture techniques may be valuable tools to increase breeding efficiency, introduce genotypes with interesting ornamental traits and shorten the time needed to obtain new cultivars. Hips resulting from a specific tetraploid *Rosa hybrida* cross were weekly harvested from 21 to 35 days after pollination (DAP). The obtained *in vitro* seedlings were multiplied using two media: M1) MS and M2) MS added with BAP (1 mg L^{-1}) and GA_3 (0.5 mg L^{-1}). The highest multiplication rate was obtained starting from embryos collected at 35 DAP on MS medium added with hormones. The shoot sprouting and the abnormal rate were generally not affected by the presence of hormones in the medium. For rooting, two media were also tested: R1) MS and R2) ½ MS supplemented with IBA (0.1 mg L^{-1}). The highest percentage of rooted explants was obtained starting from embryos collected at 28 DAP on R1 medium. The same conditions induced also the highest number of roots. The addition of IBA to rooting medium induced the development of longer roots in explants obtained by embryos collected at 35 DAP. The rooted plantlets were successfully acclimatized to *ex vitro* conditions. At flowering, the morphological traits of eight clone groups were compared with those of the seedlings obtained under common practices. *Ex vitro* plantlets showed more variability in flower colours, presence/absence of prickles, and number of petals per flower.**

INTRODUCTION

Hybrid tea roses (*Rosa hybrida*) are among the most economically important cut-flower plants worldwide. Cultivation of roses started about 5000 years ago by ancient civilizations in China, West Asia and North Africa (Gudin 2000). During history, important characteristics were introduced from the progenitors of the modern cultivars to the present gene pool (Scariot et al. 2006; De Cock et al. 2007).

Seeds are mainly used for propagation of rose species, for the production of rootstocks, and in plant breeding to obtain new cultivars (Horn 1992, Pipino et al. 2011, Pipino et al. 2013, Bosco et al. 2015). Ornamental roses are usually propagated by budding or bench-grafting onto rootstocks (Short and Roberts 1991). However, these methods are very slow, time consuming and may be a limitation in stock plants. Alternative techniques of rose propagation are needed to minimize these problems.

In vitro culture is becoming increasingly popular as an alternative faster mean of plant propagation, yielding large numbers of self-rooted plants in a very short time. Micropropagated plants are suitable for cut flower production as they are more compact (Onesto et al. 1985), produce more branches and yield higher number of flowers (Reist 1985). Multiplication of a variety through shoot tip, axillary bud and nodal segments could allow to obtain disease free plants and maintain genetically pure, healthy and vigorous stock plants (Ozen and Arslan 2006). *In vitro* techniques may be also used to improve rose breeding programs (Gudin 1994, Caser et al. 2014). New variability was obtained by several different explants such as axillary buds, shoot tips, callus, anthers, and embryos (Bressan et al. 1982; Carelli and Echeverrigaray 2002; Nikbakht et al. 2005; Ram et al. 2011).

The present study reports the *in vitro* multiplication of the offspring of a rose cross, the acclimatization of the cloned plantlets and the evaluation of the plant variability obtained by this protocol.

MATERIALS AND METHODS

Plant material

Manual pollinations were performed in the greenhouse of the NIRP International (Bevera, Ventimiglia, Italy) in summer 2010. Rose hips obtained from a cross of tetraploid *Rosa hybrida* commercial cultivars (code 1985 x code 1848) were harvested at 21, 28, and 35 days after pollination (DAP) and immature seeds and the extracted embryos were used for *in vitro* germination assays as reported by Caser et al. (2014). Fifteen vigorous *in vitro* germinated seedlings were chosen for multiplication and rooting trials.

In vitro multiplication

Apical shoots, 2 cm in length, were placed vertically on multiplication medium in five glass vases. Each vase contained five shoots which represented a replication for a total of 25 explants for each treatment. Two different media were tested for inducing adventitious shoots: (M1) MS macro, microelements and vitamins, sucrose (30 g L^{-1}), Gelrite (4 g L^{-1}) with a pH of 5.7 and (M2) M1 supplemented with BAP (1 mg L^{-1}) and GA_3 (0.5 mg L^{-1}). Growth parameters such as multiplication rate (total number of shoots divided by the initial number of explants), shoot sprouting frequency (the ratio of shoot sprouting to total shoot), and abnormality index (the ratio of abnormal to normal shoots) were calculated. Plantlets exhibiting undifferentiated growth and deformities were considered abnormal.

In vitro rooting

Plantlets were placed on two rooting media: (R1) MS macro, microelements and vitamins, sucrose (30 g L^{-1}), Gelrite (4 g L^{-1}) with a pH of 5.7 and (R2) ½ MS macro, microelements and vitamins, sucrose (30 g L^{-1}), and Gelrite (4 g L^{-1}) supplemented with IBA (0.1 mg L^{-1}). Growth parameters such as percentage of rooting, number of produced roots, and root length were considered. For both multiplication and rooting phases, explants were maintained for 4 weeks in the growth chamber with 60 % U.R. and a 16-h photoperiod at 55 µmol $m^{-2}s^{-1}$ under a cool, white fluorescent lamp.

Plant acclimatization

The *in vitro* plantlets with a well developed root system were transferred to *in vivo* conditions for the acclimatization phase. Each plant was placed in autoclave-sterilized soil (peat : perlite; 50 : 50), under 100% humidity conditions for 15 days. Acclimatized plants were then transferred into 14 cm diameter pots (peat 50 : perlite 50) and maintained in a CRA-FSO (Sanremo, Imperia, Italy) greenhouse with 70 % relative humidity, under natural light. Irrigation was applied twice a week to maintain adequate moisture. Morphological parameters were evaluated to describe the phenotypic variability shown by the plants obtained through *in vitro* techniques compared with the seedlings of the same cross obtained through the typical germination practices. We examined flower-related traits, such as number of petals per flower and colour compared to that described by the Horticultural Colour Chart of The Royal Horticultural Society, London, England (RHS Colour Chart). The number of prickles per internode was also recorded and their presence was described according to the following classes: 0 = no prickles; 1 = small not sharply prickles; 2 = small sharply prickles; 3 = big sharply prickles; 4 = presence of several small and big sharply prickles. Furthermore, the number of days elapsed from pollination to first blooming was counted.

Statistical analysis

Arcsine transformation was performed on all percent incidence data before statistical analysis in order to improve homogeneity of variance. For all parameters, mean differences were computed using a one-way ANOVA with REGW-F posthoc test ($P \leq 0.05$). All analyses were performed with SPSS 17.0 Inc. software (USA). Principal Component Analysis (PCA) was performed on morphological traits. The first two axes were plotted according to the extracted Eigen vectors, using the software package NYSYS-pc version 2.1 (Applied Biostatistics Inc., NY, USA).

RESULTS AND DISCUSSION

In vitro differentiation of adventitious buds was evaluated on seedlings germinated from plant material harvested at 21, 28, and 35 DAP (Table 1). The highest multiplication rate was obtained culturing explants obtained by embryos collected at 35 DAP on MS added with plant growth regulators (BAP and GA_3) with 5.28 shoots per explant. The shoot sprouting and the abnormal rate were generally not affected by the presence of hormones in the medium. Differences were noted only in seedlings obtained by embryos collected at 21 DAP, which showed higher shoot sprouting (100%) and lower abnormal rate (4%). Shoots were then placed on rooting media (Table 2). Statistical differences were observed in rooting percentages among the media. At 28 DAP was observed the highest frequency in R1 (16%). The same conditions induced also the highest number of roots. Root length was not affected by the presence of plant growth regulators. Overall, explants obtained by hips collected at 35 DAP developed longer roots on media supplemented with hormones, as opposed to those collected at 21 and 28 DAP (2.68 cm, 2.00 cm and 1.20 cm, respectively). The optimization of *in vitro* propagation protocols is laborious. A wide variability on *in vitro* multiplication within the genus *Rosa* was previously reported. Horn (1992) indicated significant genotype effects on *in vitro* multiplication and rooting in different cultivars of Floribunda and Hybrid tea rose. Khosh-Khui and Sink (1982) observed different rate of shoot multiplication ranging from 5.5 shoots per explants in the cv 'Bridal Pink' to 3.6 in *R. canina*. The same authors reported variability also in rooting ability of different roses (*R. hybrida*, *R. canina* and *R. damascena*). Moreover, the application of growth regulators can influence rose

multiplication (Kirichenko et al. 1991). In *R. hybrida* the inclusion of BAP (1.0 to 10.0 mg/l) was essential for shoot multiplication (Bressan et al. 1982). Rout et al. (1990) observed that media supplemented with BAP and GA_3 at low concentration induced early bud break and enhanced rates of shoot multiplication with more than seven shoots per explants in hybrid roses.

After multiplication and root induction, plantlets were transferred to soil and acclimatized under greenhouse conditions. *In vivo* acclimatization of *ex vitro* plantlets is a delicate and variety-depending phase (Davies 1980). A rapid desiccation of plantlets or their susceptibility to diseases due to high humidity can occur (Messenguer and Mele 1986). In this study new leaves started to develop after a few days. Overall, a total of 58.8% of rooted plantlets acclimatized well (data not shown). Eight clone groups were chosen among the acclimatized plants and their origin is reported in Table 3. Five morphological and blooming characteristics were evaluated as quantitative and multistate traits. Mean, maximum, minimum, and standard error of the quantitative traits are shown in Table 4. The observed diversity indicated a high level of variation among genotypes. The mean diversity calculated was 0.350 and the Shannon index was 0.571. Specifically, the characteristics of each clone group are described in Table 5. Comparison within groups revealed that all analysed plantlets demonstrated at least one variation. RhC4 was the group with the highest number of clones (11), Shannon index (1.170), and diversity (0.579), while RhC2 had the group with the lowest Shannon index (0.347) and diversity (0.222). Only one clone was obtained for RhC6. In order to visualize how *in vitro* culture influenced phenotypic variability, a Principal Component Analysis (PCA) was performed on five traits of *ex vitro* plantlets (RhC1, RhC2, RhC3, RhC4, RhC5, RhC6, RhC7, and RhC8), *in vivo* seedlings (15 plants obtained at NIRP International using a common sowing technique), the mother plant (code 1985), and the pollen donor (code 1848). The attributes responsible for maximum separation along the first two components within each clone group are reported in Table 5. Three out of five traits were individuated as principal components: days from pollination to flowering, number of prickles per internode, and number of petals. The PCA showed that the first three components accounted for 63.30%, 20.94%, and 11.30% of the variance, respectively, and the cumulative variance was 95.53%. On the basis of the eigenvector values for traits along the first two components, the attributes responsible for maximum separation were (values in parentheses) the number of prickles (8.6e-02) along the first component (PC1) and the number of petals (0.926) along the second (PC2). The first two components were used to visualize a scatter plot (Fig. 1). A wide variability of *ex vitro* plantlets was observed with a more merged distribution of *in vivo* seedlings. In particular, PCA was able to define two groups in which the studied plant morphological traits were mainly related to the mother plant (code 1985) (cluster A) or to the pollen donor (code 1848) (cluster B). In cluster A were grouped RhC4 and most of the *in vivo* seedlings, while in cluster B were grouped RhC8 and the rest of the *in vivo* seedlings. *Ex vitro* plantlets (RhC1, RhC2, RhC3, RhC5, RhC6, and RhC7) were spread on the plot. Histograms in Figure 2 show the frequency of genotypes belonging to *ex vitro* plantlets and *in vivo* seedlings and mother plant (code 1985), and pollen donor (code 1848) relatively to several factors: days from pollination (DAP) to first flowering (Fig. 3A), prickle classes (Fig. 3B), number of prickles per internode (Fig. 3C), number of petals per flower (Fig. 3D), and petal colours (Royal Horticultural Society-Chard) (Fig 3E). Differences among genotypes were observed. For both parent plants, first flowers bloomed at ca 355 DAP. The mother plant (code 1985) presented a low number (≤ 4) of small and large, sharp prickles per internode and white

(155A) flowers with petal counts ranging from 10 to 19. The pollen donor (code 1848) showed a range of 5-9 large sharp prickles per internode and yellow (8C) flowers with petal counts between 30 and 39. *In vivo* seedlings obtained at the NIRP flowered for the first time after ca 355 DAP as did parent plants, but showed variability in other traits. More than half of seedlings (57%) showed no or a low number of prickles (classes 0-1) and 29% had an intermediate number of petals per flower (20-29). Petal colours ranged from yellow (14A) to white (155A) with different intermediate red flowers. *Ex vitro* plantlets showed more variability, with 87.5 % of the total observed traits versus 50% of *in vivo* seedlings. Eighty-eight percent of acclimatized plantlets flowered earlier, with the first flowering observed at 310 DAP. They produced more prickles (50% of plantlets had more than 10 prickles per internode) and flowers with more petals (20% of *ex vitro* plantlets presented more than 40 petals). The petal colour range was wider: 9% of plantlets had green (157A) and 27% had orange (27A) flowers.

In conclusion, the applied protocol developed here to *in vitro* germinated seeds could allow breeder to obtain a higher number of cloned plants per seedling in less time, helping to shorten the time for selection. The obtained plantlets morphologically differed from the traditional offspring obtained at NIRP International. The observed variability could be useful for breeders looking for different flower colours, absence of prickles, and a high number of petals.

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Tables

Table 1. Effect of *in vitro* cultivation of *Rosa hybrida* seedlings on multiplication rate, shoot sprouting frequency (%), and abnormal rate (%) when cultured on two media. DAP means 'days after pollination' when the seeds were collected.

	Multiplication rate			Shoot sprouting frequency (%)			Abnormal rate (%)		
DAP	M1	M2	*P*	M1	M2	*P*	M1	M2	*P*
21	1.16	3.84 b[§]	**	92	100	*	17 a	4	*

28	1.16	3.36 b	**	96	100	ns	0 b	4	ns
35	1.32	5.28 a	**	100	100	ns	0 b	7	ns
P	ns	*		ns	ns		*	ns	

The statistical relevance of Between-Subjects Effects' tests (*=*P*<0.05, **=*P*<0.001, ns=non significant). §Mean values showing the same letter are not statistically different at *P*≤0.05 according to the REGW-F test.

Table 2. Percentage of rooted explants, number of roots *per* explants, and root length (cm), obtained by *in vitro* cultivation of *Rosa hybrida* seedlings on two media. DAP means 'days after pollination' when the seeds were collected.

	Rooting (%)			Roots (n.)			root length (cm)		
DAP	R1	R2	*P*	R1	R2	*P*	R1	R2	*P*
21	4b	4	ns	0.08c	0.32a	*	1.05	2.00b	ns
28	16a	8	*	0.56a	0.28a	*	1.90	1.20b	ns
35	8b	8	ns	0.28b	0.24b	ns	1.22	2.68a	ns
P	*	ns		*	*		ns	*	

The statistical relevance of Between-Subjects Effects' tests (*=*P*<0.05, **=*P*<0.001, ns=non significant). §Mean values showing the same letter are not statistically different at *P*≤0.05 according to the REGW-F test.

Table 3. Origin of the studied eight clone groups as reported in Caser et al. (2014).

Clone group	Days After Pollination (DAP)	Explant type	Germination medium	Growth condition
RhC1	21	Seed	MS	E
RhC2	21	Embryo	MS	E
RhC3	28	Embryo	MS	E
RhC4	28	Embryo	MS	F
RhC5	28	Embryo	MS	F
RhC6	28	Embryo	MS	F
RhC7	28	Embryo	MS	F
RhC8	28	Embryo	MS	F

MS= MS macro, microelements and vitamins, sucrose (30 g L^{-1}), Gelrite (4 g L^{-1}) with a pH of 5.7 supplemented with BAP (2.5 mg L^{-1}) and GA_3 (0.5 mg L^{-1}). E= one week dark period at 4°C followed by a 16-h photoperiod for all the experiment at 23°C. F= one week dark period at 4°C followed by a 16-h photoperiod for all the experiment at 15°C.

Table 4. The 5 descriptors observed in *ex vitro Rosa hybrida* plantlets.

Characteristics	Type	Mean	Minimum	Maximum	Standard error
Petals per flower (n)	Quantitative	21	5	67	3.05
Prickles per internode (n)	Quantitative	11	0	22	1.23
Days from pollination to flower (n)	Quantitative	307	289	330	2.24
Flower color	Multistate				
Prickles presence	Multistate				
Shannon Index	Diversity (range)				
0.571	0.350 (0.200 – 0.579)				

Table 5. Code, number of clones, Shannon index, diversity, and attributes responsible for maximum separation along the first and second components (PC1 and PC2) with eigenvectors in parentheses for each clone group of *Rosa hybrida*.

				Attributes (eigenvectors)	
Clone group	n°	Shannon index	Diversity	PC1	PC2
RhC1	5	0.953	0.552	Days to 1st flower (-0.003)	Prickles number (0.926)
RhC2	3	0.347	0.222	Days to 1st flower ($5.9^{e-0.4}$)	Prickles number (0.234)
RhC3	2	0.416	0.300	Petals number (-0.030)	Prickles number (0.257)
RhC4	11	1.170	0.579	Prickles number ($7.7^{e-0.4}$)	Petals number (-0.933)
RhC5	2	0.277	0.200	Days to 1st flower (-0.005)	Prickles number (0.533)
RhC6	1	-	-	-	-
RhC7	2	0.416	0.300	Days to 1st flower (0.034)	Petals number ($9.1^{e-0.4}$)
RhC8	2	0.416	0.300	Days to 1st flower (0.007)	Petals number (-0.021)

Figures

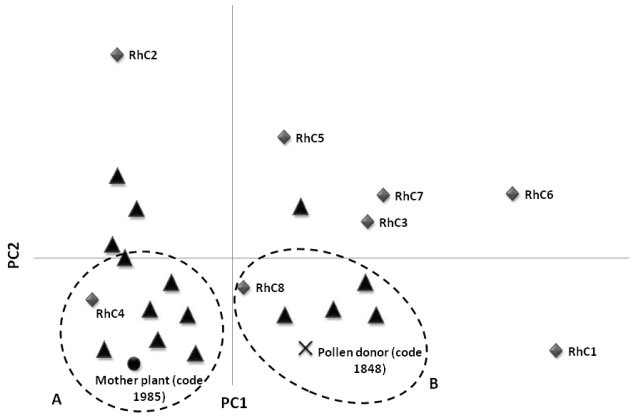

Figure 1. Scatter diagram of *ex vitro* rose plantlets (8 clone groups in grey rumbles), 15 seedlings (in black triangles), the female parent (code 1985) and the male parent (code 1848) obtained performing the Principal Component Analysis (PCA) on five morphological characteristics related to flowers, petals, and prickles. The first two components explain 84.24% of the total variation.

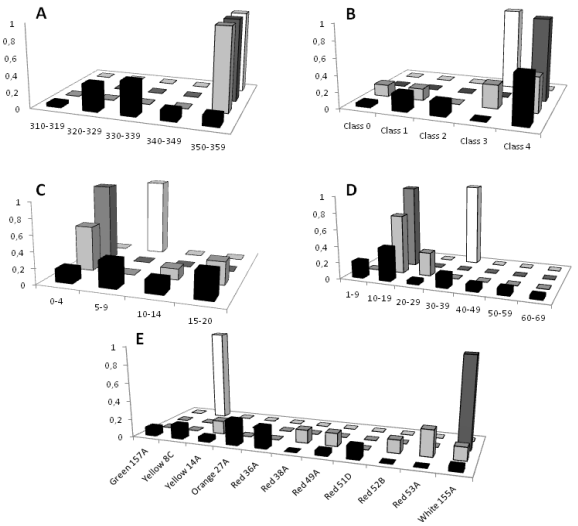

Figure 2. Histograms representing the frequency of number of days elapsed from pollination (DAP) to the first blooming (A), prickles classes (B; 0=no prickles; 1=small

not sharply prickles; 2=small sharply prickles; 3= big sharply prickles; 4= presence of several small and big sharply prickles), number of prickles *per* internode (C), number of petals *per* flower (D), and range of petal colors (E). The frequencies related to *ex vitro* plantlets, *in vivo* seedlings obtained at the NIRP, the female parent (code 1985), and the male parent (code 1848) are shown in black, light grey, dark grey, and white, respectively.